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CELL PROLIFERATION AND ELONGATION IN NORMAL, DWARF AND GIBBERELLIN- TREATED DWARF WATERMELON SEEDLINGS

PETER BAO-WEI LIU

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CELL PROLIFERATION AND ELONGATION
IN NORMAL, DWARF AND GIBBERELLIN-TREATED
DWARF WATERMELON SEEDLINGS

by

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B.S. Catholic Fu-Jen University, 1968

M.S. University of New Hampshire, 1972

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ABSTRACT

CELL PROLIFERATION AND ELONGATION
IN NORMAL, DWARF AND GIBBERELLIN-TREATED
DWARF WATERMELON SEEDLINGS

by

PETER BAO-WEI LIU

A cytological study of hypocotyl elongation in normal (Sugar Baby), dwarf (dw-2) and dwarf treated with gibberellin (GA_3) seedlings of watermelon (Citrullus lanatus (Thunb) Matsumura) revealed that reduced hypocotyl length in the dw-2 dwarf was due to fewer as well as shorter cells. However, a reduction in the cell number was the predominant factor. GA_3 normalized the growth of the mutant through an enhancement of both cell length and cell number.

The relationship between DNA synthesis and cell elongation was investigated by employing 5-fluorodeoxyuridine (FUDR), a specific inhibitor of DNA synthesis. Growth of normal, dwarf and GA -treated dwarf seedlings was completely inhibited by a 10^{-4} M concentration of FUDR. This inhibition could be reversed by appropriate concentrations of thymidine but not uridine. The results suggest that DNA synthesis is necessary for GA -induced cell elongation as well as for cell division.

The cell cycles in the shoot meristems of normal, dw-2 dwarf, and the dwarf treated with GA were compared by the labeled mitosis technique. The cell cycle was longer in dwarf than in normal plants, primarily because of a longer S period. The cell cycle in dwarf seedlings was shortened by GA treatment, and this effect was mainly attributable to the shortening of the duration of the S period, although G1 and G2 were also significantly

shorter.

An analysis of the cell proliferating kinetics in the shoot meristems indicates that only about two-thirds of the cells in the shoot meristem of dw-2 dwarf are actively dividing, virtually all of the cells in the meristematic zone of the GA-treated dwarf are actively dividing. Thus, it appears that GA promotes cell proliferation not only by decreasing the duration of the cell cycle, but also by inducing non-dividing cells in the apical meristem to divide.

INTRODUCTION

The dw-2 dwarf gene in watermelon exerts a shortening effect on hypocotyl length through a major reduction in cell number and a minor reduction in cell length. This dwarf, which can be restored to the normal phenotype at both the morphological and cellular level with exogenous applications of gibberellins (49,51), provides an excellent system for studying the action of GA on the processes of cell division and cell elongation.

The effects of GA on nucleic acid and protein metabolism in elongating cells have been extensively studied. In the elongating stems of lentil (63), soybean (33) and dwarf pea (13,14), the inhibition of RNA and protein synthesis by chemical inhibitors blocks the process of cell elongation induced by GA. Cell elongation induced by GA that depends upon DNA synthesis has been found in lentil epicotyls (63,64), cucumber hypocotyls (21), but not in soybean hypocotyls (33), and dwarf pea stems (14).

There is sparse information on the possible mechanisms of how cell division is reduced by some dwarf genes and how it is increased by administering GA to these same genetic dwarfs. One approach for studying the effect of dwarf genes and GA on cell division would be to study the cell cycle and rates of cell production in dwarf and GA-treated dwarf meristems. The duration of the cell cycle comprises four distinguishable periods

a presynthetic period (G1), a period of DNA synthesis (S), a premitotic period (G2), and mitotic proper (M). Since different metabolic programs operate in each period (62,65), the investigation of the stage or stages of the mitotic cycle which are altered by the dwarf gene and GA would provide a clue as to the cellular metabolic processes affected by the dwarf gene and GA.

There have been a few attempts to explain the effect of dwarf genes on shoot morphogenesis by comparing the mitotic activity in the shoot meristem with the rate of cell division between dwarf and normal plants (1,7). However, Burholt and Van't Hof (17) point out that there is not always a direct relationship between cell production and the mitotic index because of possible differences in the duration of mitosis between the compared materials. They also state that no single parameter, such as mitotic index, the duration of cell cycle, or the number of proliferating cells will provide useful data for the estimation of cell production, since the rate of cell proliferation is determined by the combination of all these parameters. As such, a more detailed cell kinetic analysis in the meristematic region of the shoot apex is necessary for estimating cell production in growing stems.

To obtain information on how GA acts on the processes of cell division and cell elongation, and the mechanism of the action of the dw-2 dwarf gene in watermelon, the present study was conducted with the following objectives: (1) to

determine whether cell elongation induced by GA is dependent upon DNA synthesis; (2) to compare the duration of the cell cycle and its components in a normal, and a dwarf strain of watermelon and in the dwarf treated with GA; (3) to make a detailed histological study of the shoot meristem in normal, dwarf and GA-treated dwarf watermelon.

LITERATURE REVIEW

Genetic Dwarfs

Dwarf plants caused by gene mutations affecting stem length occur commonly in higher plants. In Cucurbitaceae, genetic dwarfs have been reported in several genera, such as Cucurbita (23), Cucumis (22), and Citrullus (48). In most cases, single gene recessive inheritance is involved. Only a brief summary of the nature of dwarfism will be given here, because the subject has been extensively reviewed elsewhere (48,67).

Morphology of dwarfs. Dwarfs differ from normal plants mainly in the length of internodes. However, fewer as well as shorter internodes in certain dwarf plants have also been found (7,67). Other abnormal morphological features often associated with dwarfism include crinkled and more rigid leaves in dwarf pea (43) and watermelon (48); dark green leaves in tomato (52) and watermelon (48); brittle stems in columbine (3) and muskmelon (22); twisted stem in watermelon (48), and the dark red of vegetative part in dwarf-red upland cotton (59).

Cytology of dwarfs. Both cell division and cell elongation are usually involved in the reduction of internode length in dwarf plants. Nevertheless, the dwarfness in a number of genetic

dwarfs is mainly due to the differences in cell number (23,31, 34,51). Cell size differences usually occur during maturation but not in the meristematic region of dwarf and normal plants (1,55). Only in a few instances has the specific region of cell division been compared in dwarf and normal plants (1,7), and little has been reported about the specific effects of dwarf genes on cell division.

Gibberellin and Stem Elongation

The gibberellins represent an important class of plant hormones, noted particularly for their dramatic enhancement of stem elongation. Although a number of structurally similar gibberellins have been isolated and identified, the principal compound employed in studies of plant response is gibberellic acid (GA_3) (10,12,45,66).

Reversal of dwarfism. Brian and Hemming (11) first demonstrated that a genetically dwarf variety of pea responded more strongly to gibberellin (GA) than the normal or tall type, and the dwarf was restored to the height range of the tall variety by an appropriate dosage of the hormone. Subsequently, a number of similar results of the reversal of genetic dwarfism by GA have been reported in pea (27), corn (68), tomato (69), and cucurbits (22,50,51). It was thus suggested by several researchers that lower levels of endogeneous GA in genetic dwarfs is the limiting factor in causing the short stature of growth. Lower levels of endogeneous GA in dwarfs than in normal have been reported in maize (68), beans (70), and

Japanese morning glory (6). However, similar amounts of GA in dwarf and tall varieties have also been found in peas (40), and rice (75). Even in certain dwarfs in rice (75) which do have lower levels of endogeneous GA, there does not appear to be a direct relationship between GA level and plant height.

Cellular basis of GA action on genetic dwarfism. Cooper (19) observed that the rosette form in a dwarf mutant of perennial ryegrass was caused by the reduction of cell elongation in the leaf blade and sheath. The normalization of the dwarf mutant by the treatment with GA was primarily due to the increase of cell elongation in the leaf blade and sheath. On the other hand, Basford (7) found that the effects of GA which led to the conspicuous increase in height of dwarf groundsel plants were mainly mediated through the increase of mitotic activity in the shoot subapical meristem. In watermelon, Loy and Liu (51) reported that the elongation of dwarf hypocotyls induced by GA was mostly attributable to increased cell numbers. In most cases, the GA enhancement of stem growth in genetic dwarfs appears to be primarily due to increased cell division in the meristematic region of the shoot apex. Thus, these genetic dwarfs can provide an excellent system for studying the action of GA on cell division.

Gibberellin regulation of nucleic acid metabolism. Nitsan and Lang (63) studied DNA synthesis in elongating lentil epicotyls incubated with GA. Growth of the epicotyl was due to cell elongation alone; the cell number did not increase during this period. The cell elongation occurring in the presence of GA could be inhibited by 5-fluorodeoxyuridine (FUDR). The

inhibitory effect could be restored by thymidine, but not by uridine. They also demonstrated (64) that the GA-stimulated extension growth of lentil epicotyls was accompanied by a significant increase in DNA synthesis. So they concluded that the cell elongation induced by GA depended upon DNA synthesis. A similar experiment was also conducted by Degani et al. (21) on cucumber hypocotyl sections. They found that the inhibition of GA-induced elongation with FUDR was caused by the inhibition of DNA synthesis. Later, they (20) noted that the enhancement of DNA synthesis by GA took place outside of the cell nucleus, probably in chloroplasts and mitochondria. It has been observed in the hypocotyls of Sinapis alba that inhibition of DNA synthesis by FUDR precedes inhibition of stem elongation, thus implicating DNA synthesis in cell elongation (9).

On the other hand, GA induced cell elongation in the absence of DNA synthesis has also been extensively reported in the other types of growing tissue. Haber and Luippod (29) found that wheat, after receiving large doses of gamma rays, could germinate and grow into small seedlings without undergoing any mitosis or detectable DNA synthesis. FUDR which had been shown to be a specific and efficient inhibitor of DNA synthesis in irradiated wheat plantlets (30) did not inhibit the growth induced by GA. They concluded that GA induced the germination of such irradiated seedlings by stimulating the elongation of non-dividing cells with no effect on DNA synthesis itself. Rose and Adamson (72) obtained similar results from the growth of irradiated wheat seedlings. Kadouri et al. (41) reported that FUDR did not inhibit the GA-enhanced growth of gamma-radiated

wheat plantlets while 6-methyl purine did so very effectively. It seems that in this growth system, in which DNA synthesis does not occur owing to the irradiation pretreatment, growth depends on RNA synthesis which is inhibited by 6-methyl-purine.

Holm and Key (33) studied the hormonal regulation of cell elongation in the hypocotyls of rootless soybean. They found that cell division only occurred in the apical section; below the dividing zone, only cell elongation took place. GA induced the growth of both apical and elongating sections whereas DNA synthesis increased in the apical region but not in the elongating section. FUDR inhibited DNA synthesis in the apical section and consequently, inhibited the growth which was induced by GA. However, since there was no increase in DNA synthesis in the elongating section, FUDR did not inhibit the growth that was induced by GA.

The effects of GA on nucleic acid metabolism in dwarf pea internodes have been reported by Broughton (13,14). He measured DNA synthesis in dwarf pea stems at different times after treatment with GA. The rate of net DNA synthesis was relatively low in both treated and control plants. GA had no or little effect on DNA synthesis during the earlier stage of growth, whereas the growth was significantly enhanced by GA. Subsequently, there occurred a marked increase in DNA synthesis and the rate of growth increase in treated internodes was approximately double that of the controls. The treated internodes had a final DNA content double that of the control, which was in good agreement with the GA-induced increase in cell number. Later, Broughton found that FUDR, which completely blocked cell

division, did not prevent cells from elongating in the pea internode in vivo in the presence of GA. However, internodes treated with actinomycin D failed to elongate in response to GA treatment. He concluded that RNA, but not DNA synthesis was essential for gibberellin-stimulated cell elongation to occur in this tissue. Johri and Varner (38) observed that quantitative changes in RNA synthesis were induced by GA in dwarf pea internodes. They noted that GA enhanced the rate of RNA synthesis in the isolated nuclei of dwarf pea internodes. In GA-treated internodes the newly synthesized RNA differed in nearest neighbor frequency and size distribution from control. However, Thompson and Cleland (78), using the DNA-RNA hybridization technique, could not find the appearance of a new RNA species in light grown dwarf pea internodes treated with GA for 36 hours.

All of the above evidence suggests that the effects of GA on cell elongation may be associated with nucleic acid metabolism. Stem elongation is based on both cell division and cell elongation. Since both of these processes may be stimulated by the plant growth hormone gibberellin, the investigation of the mechanism of GA action on both cell division and cell elongation in stem growth should be equally important. However, most studies concerning the effects of GA on nucleic acid metabolism in growing tissue have focused on cell elongation, and not on how GA regulates cell division in the meristematic region of growing stems.

Biology of Cell Division

DNA synthesis and cell division. Howard and Pelc (35) first demonstrated from autoradiographic studies with cells of mouse that DNA was duplicated during a certain restricted period of interphase. This period of synthesis, the S phase, was preceded by a gap, the G1 phase, in which there was no DNA synthesis, and was followed by another one, the G2 phase, in which there was also no DNA synthesis. The G2 phase ran into mitosis (M) and the end of the cycle. Considerable evidence that DNA synthesis is a discontinuous event in the interphase of the cell division process in eucaryotes has been accumulated in the past two decades. Most of the enzymes in eucaryotic cell cultures that have been studied recently are synthesized discontinuously at a particular stage of the cycle (58,61,62). Consequently, the recognizable sequence of G1-S-G2-M has served as a convenient marker for exploring the general principles of the cell division in higher organisms.

Wimber (85) first applied ^3H -thymidine to the root tips of Tradescantia paludosa and estimated the cell population kinetics. Since then, many measurements of the mitotic cycle have been conducted on proliferating cells in the root meristems of higher plants.

Effects of DNA content and chromosome structure on the cell cycle. Van't Hof and Sparrow (84) studied the relationship between DNA content, nuclear volume and the duration of mitotic cycle in the root meristem of six unrelated species. They concluded that the higher DNA content in large interphase

nuclei prolonged the duration of the mitotic cycle through the lengthening of the S period. The correlation between the duration of mitotic cycle, interphase nuclear volume, and DNA content was constant. If any one of the three cell parameters was known, an estimation could be made of the remaining two. Van't Hof (81) also obtained from linear regression analysis a correlation of +0.97 between DNA content per nucleus and the DNA synthesis period (S). Bryant (16) measured the cell cycle in the root meristem in seedlings of three species of onion. Differences in the duration of cell cycle among the species were due to differences in the S period which were related to the DNA content. Langridge et al. (46) observed the duration of phases of the cell cycle in five species and one hybrid of Crepis (Compositae) which differed in chromosome size. The species with significantly longer chromosomes had a longer S period, and consequently, a longer cell cycle. They concluded that a positive correlation existed between the size of the genome, length of the S period and the length of cell cycle. Evans and Rees (25) derived similar results in seven unrelated species of monocotyledons and dicotyledons.

On the other hand, within the same species some studies have indicated no positive correlation between the amount of nuclear DNA and the duration of DNA synthesis period. Van't Hof (82) induced tetraploid cells in the root meristem of Pisum by blocking cytokinesis at metaphase with colchicine. Cells that did not reach metaphase during the treatment period would remain diploid, so that after removed of the colchicine

treatment, a mixed population of both diploid and tetraploid cells could be found in the root meristem. Although the mitotic cycle was prolonged by colchicine, the S period of the colchicine-induced tetraploid cells was no longer than that of diploid cells. Friedberg and Davidson (26) obtained similar results from a study of mixed cell populations in root meristems of Vicia faba. In oats, Yang and Dodson (86) compared the nuclear DNA content and the duration of DNA synthesis periods in related diploid and autotetraploid species. They reported that the durations of DNA synthesis in diploid and autotetraploid were similar. Troy and Wimber (79) also found a relatively constant durations of DNA synthesis between diploids and autotetraploids in several species of Chrysanthemum. Avanzi and Deri (4) studied the duration of the mitotic cycle in two cultivars, Aziziah and Cappelli, in Triticum durum. They noted that the shorter mitotic cycle in Aziziah as compared to Cappelli was mainly due to the shorter S period. However, the difference could not be ascribed to differences in DNA content in two cultivars, since DNA content per nucleus was even 6% higher in Aziziah than in Cappelli. The duration of the DNA synthesis period in root meristem cells of white spruce seedlings from four provenances with different DNA contents were studied by Micksche and Rollins (60). They concluded that DNA quantity did not affect the length of the DNA synthesis period and the duration of mitotic cycle.

In all instances cited above, it can be generally summarized that nuclear DNA content is not directly correlated with the duration of DNA synthesis in related species, but the correlation may exist in the unrelated species. Since the

chromosome structure is different in unrelated species, the differences in the DNA synthesis duration may also be caused by differences in chromosome structure instead of by variation in nuclear DNA content.

Taylor (77) first proved that there were many discrete sites on chromosomes which were able to initiate DNA synthesis in eucaryotic cells. Thus, different types of chromosomes in different species might vary in the number of replication units. Tanaka (76) compared the pattern of DNA replication on the chromosomes of two species in Chrysanthemum and their hybrid. The chromosomes of Ch. linear are 1.5 to 2.0 times as long as those of Ch. nipponicum. Chromosomes of the two species exhibited similar periods of duration for DNA synthesis and the mitotic cycle. In the F_1 hybrid, the DNA on homologous chromosomes of the two species replicated in synchrony. However, there were about twice as many silver grains over chromosomes of Ch. linear as the chromosome of Ch. nipponicum. The distribution of silver grains over chromosomes in both species appeared scattered over the entire length of the chromosome. The scattered appearance of the silver grains indicated multiple positions of DNA replication on each chromosome. Thus, it was postulated that there might be twice as many replication units in the chromosomes of Ch. linear as those of Ch. nipponicum.

B-chromosomes in rye are heterochromatic and replicate their DNA late at interphase. Ayonoadu and Rees (5) compared the mitotic cycle between cells in the root meristem of rye with and without B chromosome. They found that the increase in the duration of the total cycle due to the B chromosome was

paralleled by increases in the duration of all the component phases. Alfert and Das (2) studied the rate of DNA synthesis in root tip cells of diploid and autotetraploid snapdragon seedlings. They found that the doubling of the chromosome number lengthened the duration of the mitotic cycle. This increase was mainly due to an increase in the duration of G1 and S periods, while the G2 and M periods were about the same. The increase in durations of G1 and S periods in the tetraploid cells was closely correlated with the increase in nuclear surface area. Based upon the replicon hypothesis, that the initiation of DNA synthesis occurs at a specific location on the cell membrane, they suggested that the control of the rate of DNA synthesis in eucaryotes might be localized on the nuclear membrane.

Evans and Rees (25) compared the mitotic cycle and its components in several species of dicotyledons and monocotyledons. They noted, in general, that the longer mitotic cycle in dicotyledons as compared to monocotyledons was mainly due to the longer G1 period. There was no noticeable difference in the duration of either the S or G2 phase. DNA density of the chromosomes during metaphase of mitosis in dicotyledons was almost twice that in monocotyledons, and the chromosomes were more highly coiled. They suggested that the uncoiling of chromosomes in dicotyledons prior to DNA synthesis might require a longer time, thus resulting in the longer G1.

Kusanagi (44) found that the replication of DNA in barley chromosomes only occurred during one hour at the beginning of the S period and during $\frac{1}{2}$ to 1 hour before the end of the S period. The chromosomal DNA synthesis scarcely took place

in the middle of the S period. These results suggested that the replication of chromosomal DNA in barley during the S period did not proceed in a continuously temporal sequence.

The effect of chemical agents on the cell cycle. Since the control of the process of the mitotic cycle must be very exact, it can be expected that chemicals which disrupt any metabolic step during mitosis will change one or more components of the mitotic cycle.

Van't Hof (80) measured the cell cycle of excised pea roots after 72 hours in culture. The cycle duration of the cells from excised roots was longer than that of intact root meristem cells, but the duration of the S period of the cultured meristem cells was similar to that of intact root cells. Sucrose restored the normal cycle in the cells of excised roots and probably was the limiting factor. In this case, the duration of DNA synthesis remained relatively constant while the other periods of mitotic cycle were more dependent on a carbohydrate source.

The alkylating agent L-diepoxybutane induces chromatid interchanges in cells when the chemical is given before the DNA synthesis period. Matagne (57) found that mitotic cycle of root tip cells in Allium cepa was protracted by the increase of DNA synthesis phase after L-diepoxybutane treatment.

The reduction of root growth by two growth hormones, kinetin and IAA, is commonly due to decreased cell division. Van't Hof (83) observed the effect of these hormones on the mitotic cycle in the root meristem cells of pea. He found that

kinetin increased the mitotic cycle by prolonging G1 and G2, whereas the extension of mitotic cycle with IAA was mainly due to an effect on the S phase. The combination of IAA and kinetin produced all three results. Macleod (53,54) studied changes in mitotic cycle following kinetin and IAA treatment in Vicia faba and obtained similar results. In addition, he also found by comparing nuclei that both of these hormones could increase the rate of DNA synthesis, even though the hormone treatments resulted in the prolongation of the S period. This suggested that different factors might be involved in controlling the duration of the S period and the rate of DNA synthesis, since metabolic processes, other than those involved in DNA synthesis, such as histone synthesis (62), also took place in the S period.

From the above review, it is evident that there have been relatively few studies of the effects of growth regulators on the cell cycle in higher plants. In addition, cell populations of root meristems are used for most of these studies; there is sparse information on the cell cycle of apical shoot meristems.

MATERIALS AND METHODS

Plant Materials and Growing Conditions

Seedlings of two strains of watermelon (Citrullus lanatus (Thunb.) Matsumura) were used for the experiments; a dw-2 dwarf inbred line designated WB-2 (48), and a normal cultivar, Sugar Baby (SB) (Joseph Harris Co., Inc., Rochester, N.Y.)

In all experiments plants were grown in a growth chamber maintained at $30 \pm 1^{\circ}\text{C}$ under continuous yellow light at an intensity of $300 \mu\text{w}/\text{cm}^2$. The yellow light was achieved by filtering light from four 40W, cool white, fluorescent lamps through yellow cellophane and a Corning 1-75 infrared filter. The maximum differential in hypocotyl elongation between the dwarf and normal strains was obtained with this light regime. Mechanically scarified seeds were germinated in plastic germinating trays ($28 \times 41 \times 13 \text{ cm}^3$) on a layer of cellucotton moistened with distilled water. On the third day of germination, uniformly germinating seedlings were selected and transferred to disposable Petri dishes on a layer of cellucotton saturated with 20 ml. of the appropriate solution for each treatment. The roots of each seedling were carefully inserted underneath the cellucotton so that roots would develop properly without becoming desiccated. The Petri dishes were placed in plastic germinating trays which were layered with water to maintain high humidity.

Chemicals

All the chemicals which were used in the following experiments were applied in aqueous solution for each treatment in the Petri dishes

Gibberellin A₃ (K and K Laboratory, Plainview, N. Y.)

Thymidine and Uridine (Nutritional Biochemical Corporation, Cleveland, Ohio)

5-Fluorodeoxyuridine (FUDR) (Hoffman-La Roche Inc., Nutley, N. J.)

Radioactive ³H-thymidine (International Chemical and Nuclear Corporation, Irvine, Calif.)

Seedling Growth

Seedlings of the normal strain, SB, were grown in different concentrations of FUDR in an attempt to produce dwarf phenocopies by the inhibition of thymidylate synthetase activity. For each concentration, 6 seedlings were used per Petri dish. Measurements were taken at four successive 24 hour intervals beginning 18 hours from the treatments. Different concentrations of GA₃, thymidine and uridine were tested for their ability to normalize FUDR dwarfs. Six seedlings were used per treatment and measurements were taken 96 hours from the treatments. All the treatments were replicated twice.

Cytological Studies

Cell number and length in hypocotyl. Four treatments were used for this study: SB control, WB-2 control, WB-2 treated with 3.2×10^{-4} M GA_3 , and SB treated with 10^{-4} M FUDR. Seedlings were germinated for 72 hours and then were transferred to the treatment solutions for an 18 hour period (initial stage). Following this, seedlings were transferred to Petri dishes containing distilled water and allowed to develop prior to analysis. Three seedlings in each treatment were killed and fixed in Craf II at 72 hours and 162 hours after germination for the initial and final stages respectively, followed by standard paraffin embedding procedures (73). Longitudinal sections, 20 μ thick, were cut and stained with fast green. A vertical row of consecutive cortical parenchyma cells were counted under an ocular micrometer throughout the length of the hypocotyls of each seedling.

Radioisotope labeling and autoradiography. The ^3H -thymidine, 1 mCi/ml (specific activity 14.1 Ci/mM), was diluted to 500 $\mu\text{Ci}/\text{ml}$. Ten μl of the dilution (5 μCi) were carefully applied with a microsyringe to the apex of each seedling. After a one hour period of labeling, the plants were washed and returned to the cool solution. Following the incorporation of ^3H -thymidine, meristematic apices, 1 cm long, were excised and fixed in Carnoy's solution, dehydrated with tertiary butyl alcohol, embedded in paraplast and sectioned 8 μ thick. The sections were stained using the Feulgen reaction (47), and then were covered with Kodak AR-10 autoradiographic stripping film. After drying, they

were stored in a refrigerator for an exposure period of 21 days. The sections were then developed in Kodak D-19 developer, and fixed in Kodak fixer. DNA synthesis could be determined by examining the autoradiographic specimens and counting the silver grains on the nuclei.

Determination of the mitotic cycle. Based on the methods of Quastler and Sherman (71), the mitotic cycle duration (CT) and its component parts, G₁, S, G₂, and M were determined by the percent of labeled mitoses in the cells of the apical meristem during a sequence of 12 time intervals of 3 hours each. The comparison of cell cycles was conducted among dwarf, normal and GA-treated dwarf seedlings. Three seedlings per treatment were examined in each time interval beginning 18 hours from the treatments. Three median sections, 32 microns apart, were examined in each apex. The cells which were labeled at the mitotic phase according to the autoradiographic examinations were judged to be synthesizing DNA during the period of ³H-thymidine incorporation. The earliest samples should have no labeled mitoses if the G₂ period was longer than the time after pulse labeling. The proportion of labeled mitoses rises to a peak as the cells which were in S at the time of pulse come through to division. Following this peak, there is a trough as the cells originally in G₁ come to the end of their cycle. The next cycle showed a similar but depressed peak. The mitotic cycle (CT) was measured from the 50% point of maximum intercept of the two ascending portions of the curves for the percent of labeled mitoses. The period, G₂+ $\frac{1}{2}$ M, was

determined by the interval of the time from pulse labeling to the 50% intercept of the first ascending portion of the curve. The S period was the interval between the two 50% intercepts of the ascending and descending portions of the first peak. Mitotic index (MI) is the percentage of meristematic cells in mitosis. It is assumed that all of the cells in the meristematic region being examined are proliferating. The duration of mitosis (dM) can be calculated as follows:

$$dM = \frac{MI \times CT}{100}$$

The proportion of all dividing cells in prophase (%P) = dp/dM , so that the duration of prophase (dp) = %P x dM.

Similarly, the duration of metaphase (dm) = %M x dM, and the durations of anaphase and telephase (da) = %A x dM.

The G1 period can be derived from the equation, $G1 = CT - G2 - S - M$. Thus, the whole mitotic cycle and its components can be determined

The rate of cell proliferation (R) in the meristematic region can be obtained from the equation, $R = \frac{NCT}{CT}$, where NCT = the number of meristematic cells in the apical population. Also, the number of proliferating cells per file (R_f) can be derived from the equation, $R = \frac{NCT}{N_f \times CT}$, where N_f = the number of files in the apical meristem.

RESULTS

Inhibitory Effects of FUDR on Seedling Growth

Growth curves for inhibition of SB hypocotyls under different FUDR concentrations are shown in Figure 1. The growth of SB seedlings was inhibited about 55% that of control by 10^{-5} M to 5×10^{-5} M concentrations of FUDR, whereas 90% inhibition was obtained when the FUDR concentration was 10^{-4} M or higher. A FUDR concentration of 10^{-4} M was used in the subsequent experiments for inhibiting seedling growth in both SB and WB-2.

Figure 2 illustrates the reversion of FUDR inhibition by thymidine. Thymidine at 10^{-4} M started to sharply reverse the inhibitory effects of FUDR in both SB and WB-2. At a concentration of 10^{-2} M in SB and 10^{-3} M in WB-2, thymidine almost completely reversed the FUDR inhibition of seedling growth. Treatment of SB and WB-2 seedlings with thymidine at concentrations as high as 10^{-2} M did not enhance growth. Uridine at a wide range of concentrations failed to reverse any of the inhibitory effects of FUDR on seedlings of SB or WB-2.

The effect of FUDR on GA-induced growth was also studied (Figure 2). The growth of FUDR treated seedlings of SB and WB-2 under a range of GA concentrations was similar to growth in absence of GA. Thus, GA could not induce growth in normal and dwarf seedlings which were inhibited by FUDR. Thymidine could counterbalance the inhibitory effects of FUDR,

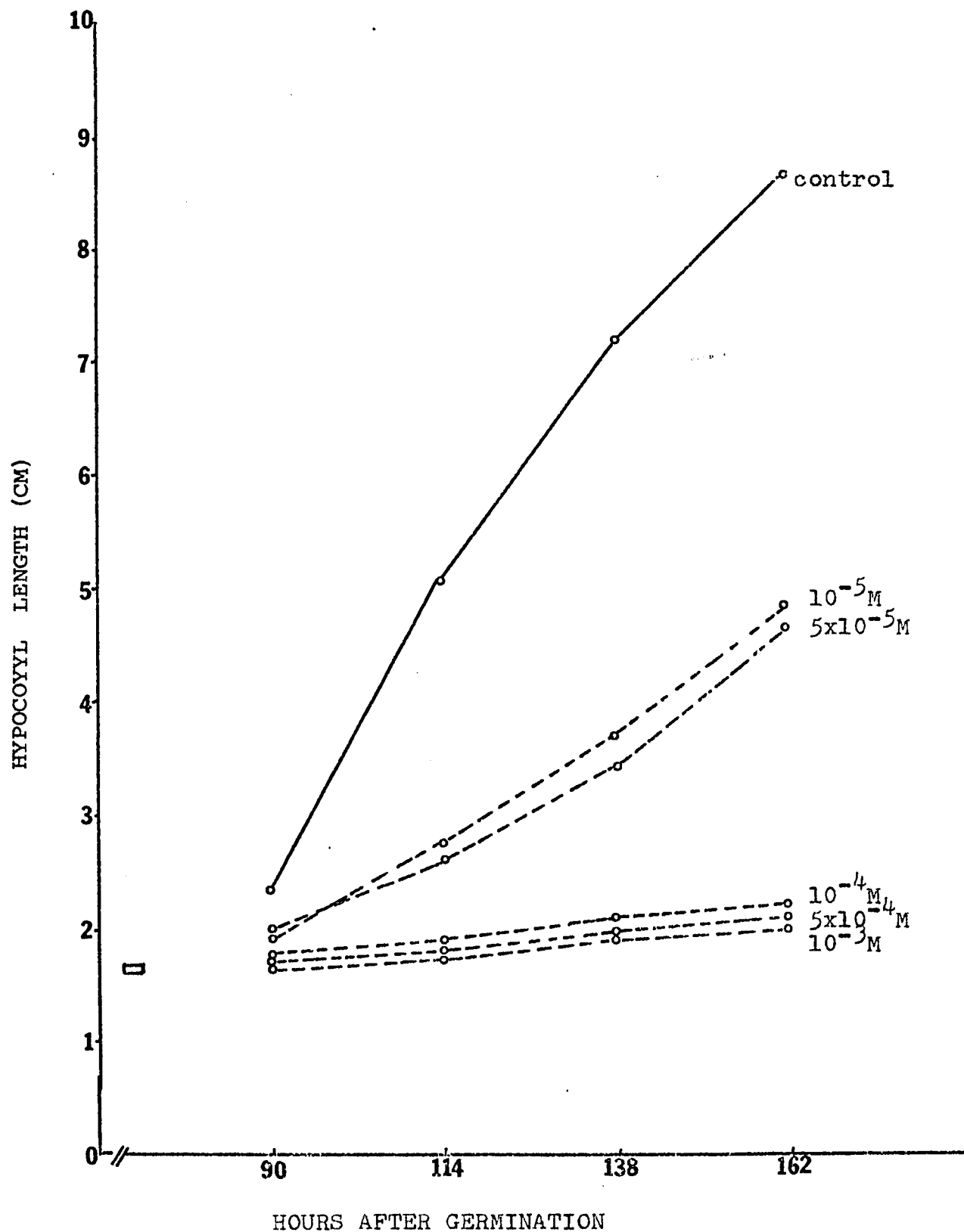


Figure 1. Growth curves of SB seedlings under different concentrations of FUDR.

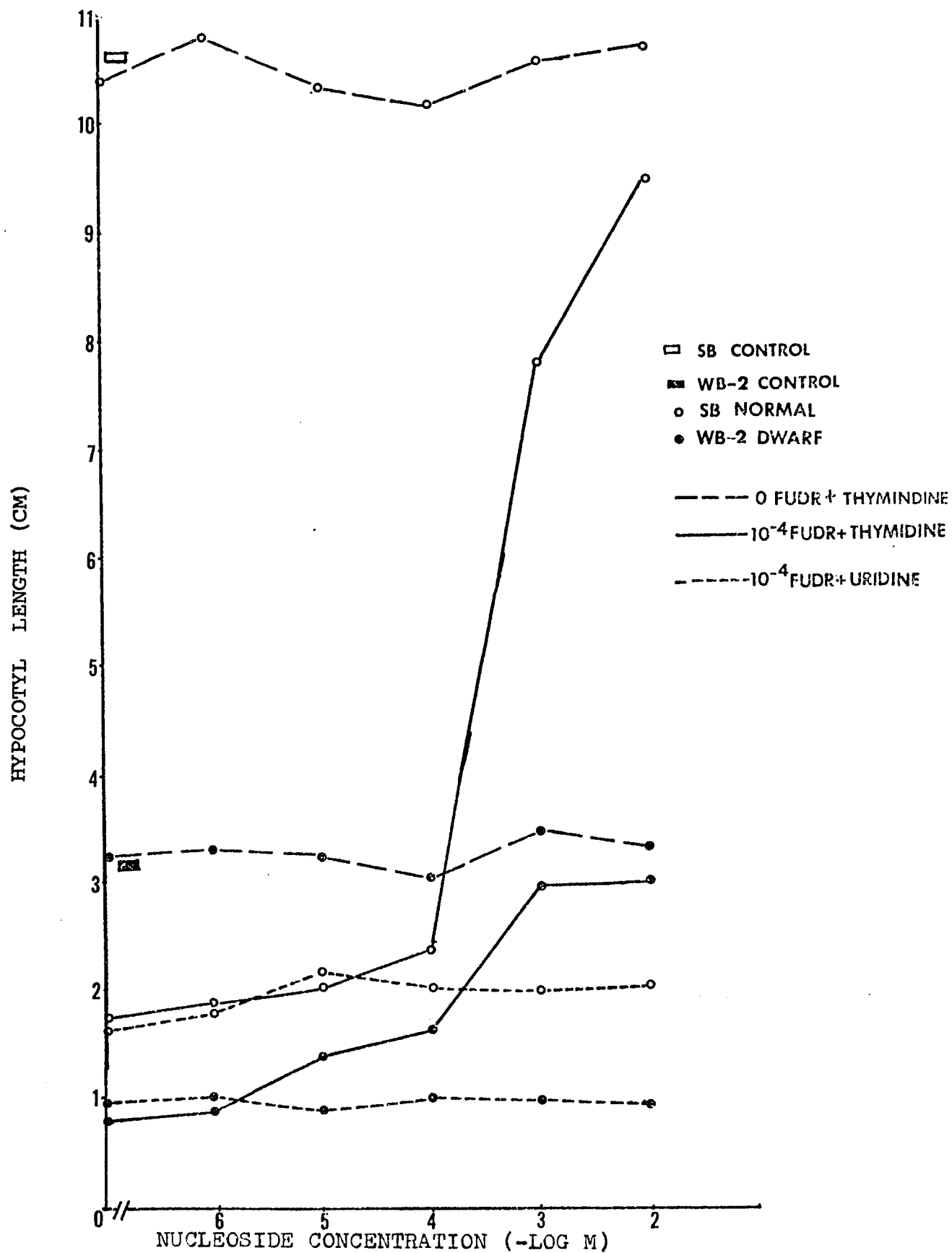


Figure 2. The effect of FUDR and added thymidine or uridine on the growth of SB and WB-2 seedlings.

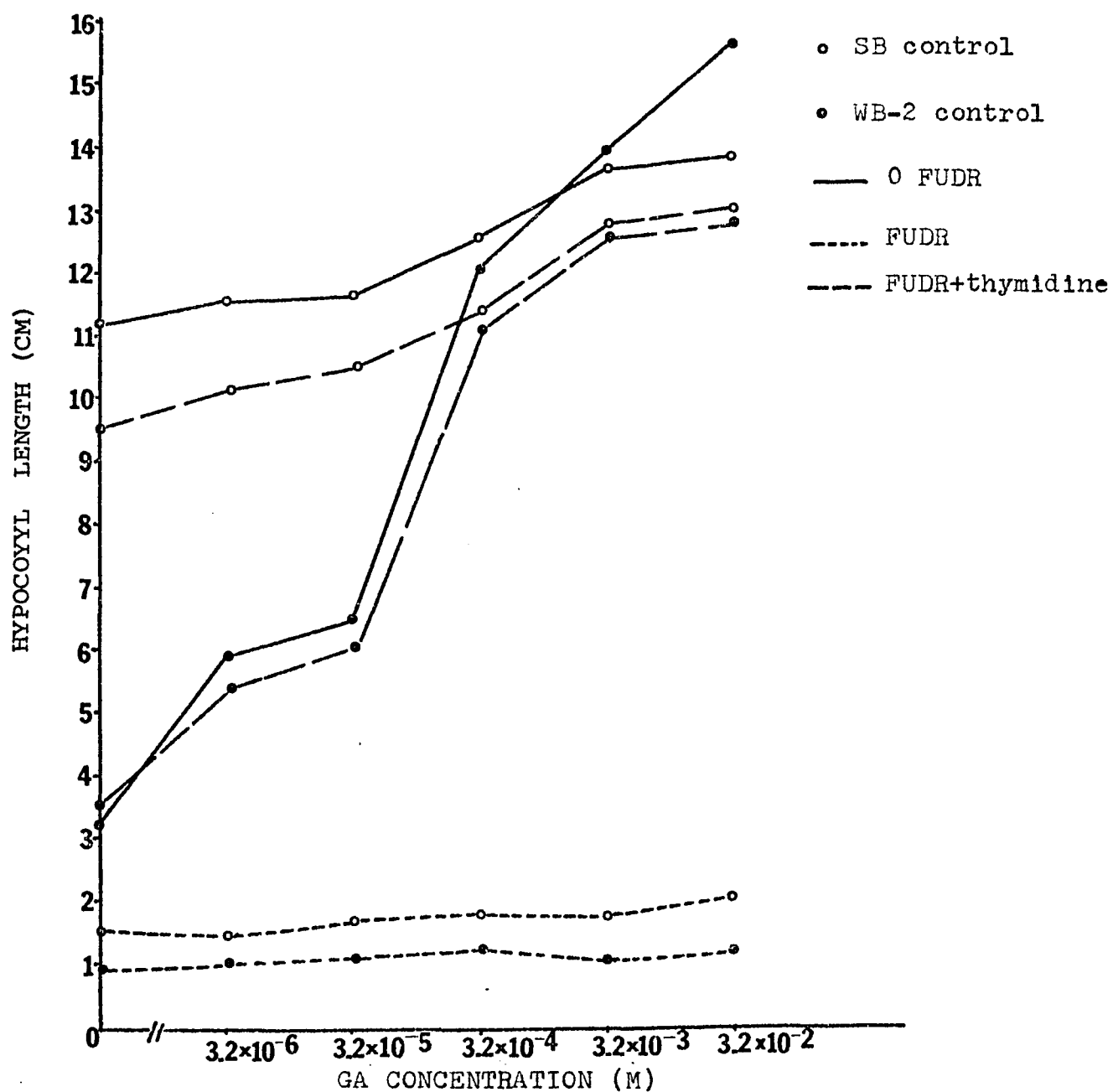


Figure 3. The effect of GA on the growth of SB and WB-2 seedlings in the presence and absence of FUDR.

and permitted the promotion of growth by GA in SB and WB-2 seedlings.

Cell Number and Length in Seedlings

The rates of growth among normal, dwarf, GA-treated dwarf, and FUDR-treated normal seedlings were compared (Figure 4). The increases in growth after 90 hours from the beginning of the treatment period were 6.3 cm in SB control, 7.1 cm in WB-2 treated with GA, 1.3 cm in WB-2 control, and 0.5 cm in SB treated with FUDR. The growth rate of GA-treated WB-2 seedlings was the fastest among four treatments whereas FUDR almost completely inhibited the growth of SB seedlings. A comparison was made of total numbers in a representative longitudinal file in the four treatments after 90 hours, (Figure 5). FUDR entirely blocked the process of cell division, since the mean cell number in SB seedlings treated with FUDR for 90 hours was no more than that of SB control at the initial stage. The average lengths of the most mature cells in SB seedlings were 212.5μ ($3400 \mu/16$). Ninety hours after treatment with FUDR, the length of the most mature cells in SB increased only from 106.3μ to 117.2μ ($3400 \mu/29$), so that about 90% of the cell elongation in SB was inhibited by FUDR.

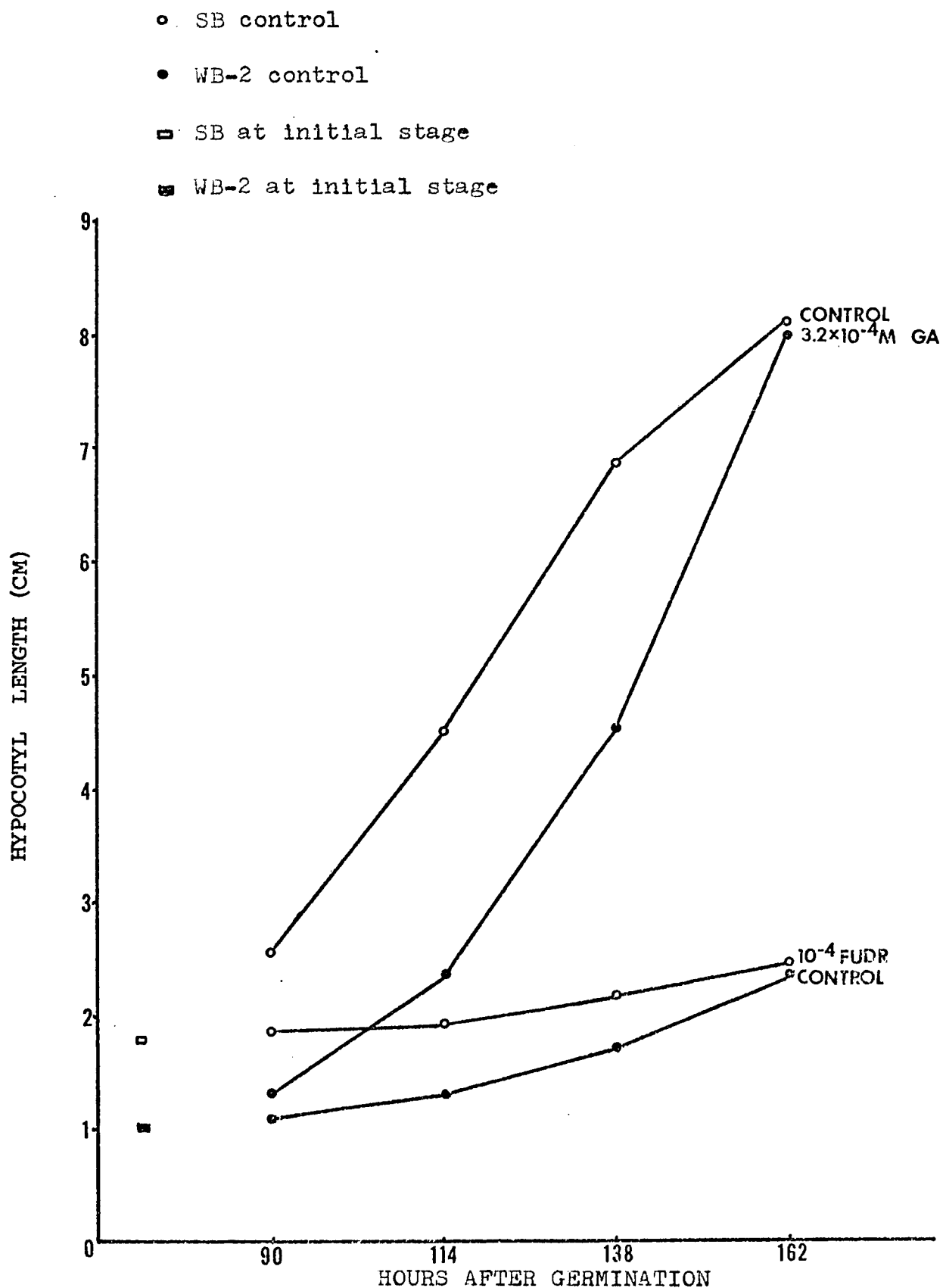


Figure 4. The growth rates of SB and WB-2 seedlings under FUDR and GA treatments.

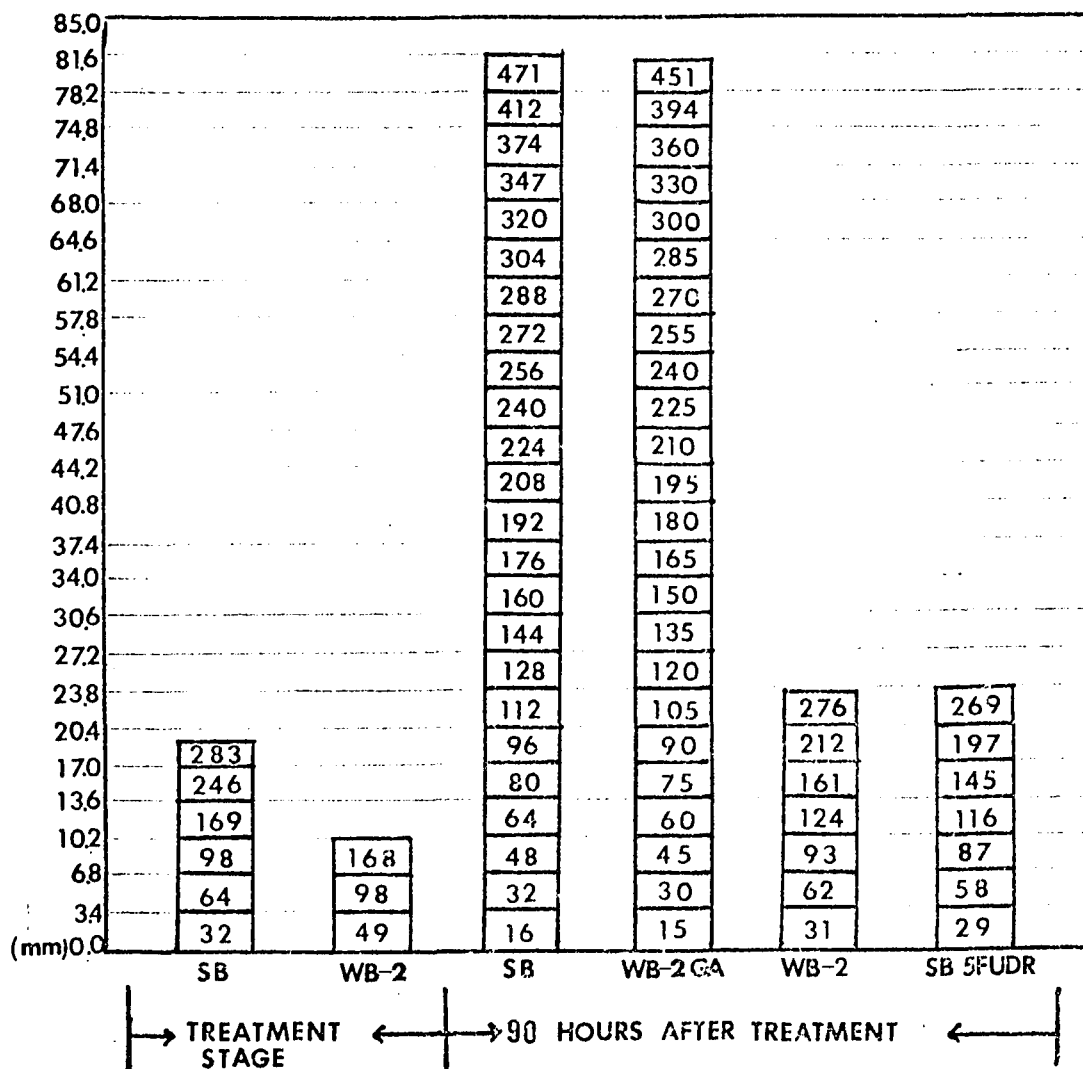


Figure 5. A diagrammatic representation of cell number and cell length in the SB and WB-2 seedlings at the beginning and at the end of a 90 hour period of growth in the presence and absence of 3.2×10^{-4} M GA in WB-2 and with and without 10^{-4} M FUDR in SB. The numbers represent cumulative cell counts per unit (1 unit = 3.4 mm) in an acropetal direction.

The total cell number per file in SB averaged 283 at the initial stage and reached 471 after 90 hours of growth. Thus, approximately 200 cells were produced during this period. During the same 90 hour period, the cell numbers were increased by 108 in the WB-2 dwarf, and by 283 in WB-2 seedlings treated with GA. Thus, the rate of cell proliferation in dwarf seedlings was increased approximately 3 times by GA treatment. After 90 hours, the lengths of the most mature cells were 109.7μ ($3400 \mu/31$) in WB-2 control and 226.7μ ($3400 \mu/15$) in WB-2 treated with GA. Mature cells were of comparable lengths in SB control and GA-treated WB-2 seedlings. The cell numbers in the first apical unit after 90 hours treatment were 59 cells in 3.2 mm in SB, 57 cells in 3.1 mm in WB-2 treated with GA and 64 cells in 3.2 mm WB-2 control, so that the cell sizes in the apical shoot meristem at this stage were 54.2μ in SB, 54.2μ in WB-2 treated with GA, and 50.0μ in WB-2. At the initial stage, the cell numbers in the first apical unit were 37 cells in 1.8 mm in SB and 70 cells in 3.4 mm in WB-2, so that the cell sizes in apical meristem of both SB and WB-2 were 48.6μ . The mean length of meristematic cells in SB treated with FUDR was 47.2μ which was similar to the size of meristematic cells in SB at the initial stage. It is clear from these results that GA and the dwarf gene affect the elongation of cells during maturation, but do not affect cell length in the apical meristems of watermelon shoots.

Cell Cycle Apical Meristem of Watermelon Seedlings

The mitosis-labeling curves for SB control, WB-2 control, and GA-treated WB-2 seedlings are shown in Fig. 6. The first peak of WB-2 control was wider than either SB control or WB-2 treated with 3.2×10^{-4} M GA_3 . The second peaks of SB control and WB-2 treated with GA appeared earlier than that of WB-2 control. The durations of the cell cycle and components of the cell cycle among the three treatments as calculated from the pulse-label curves, are given in table 1.

Table 1. Duration in hours for each of the cell cycles.

<u>TREATMENT</u>	<u>INTERPHASE</u>					<u>MITOSIS</u>		
	<u>C</u>	<u>G1</u>	<u>S</u>	<u>G2</u>	<u>F</u>	<u>M</u>	<u>A&T</u>	<u>DM</u>
SB control	21.3	5.3	9.5	5.83	.30	.23	.14	.67
WB-2 control	27.5	7.25	12.5	7.25	.21	.17	.12	.50
WB-2(GA)	18.5	4.7	7.4	5.73	.29	.24	.14	.67

The duration of the cell cycle in WB-2 control was 6.2 hours longer than SB and 9.0 hours longer than WB-2 treated with GA. The durations of mitosis and its components, prophase, metaphase, and anaphase and telephase were similar among the three treatments. The differences in the duration of cell cycle were completely due to variations in the durations of the periods in interphase. GA decreased the duration of the G1 period in WB-2 dwarf by 2.5 hours to a stage which was comparable to the duration of G1 in the SB control. GA also

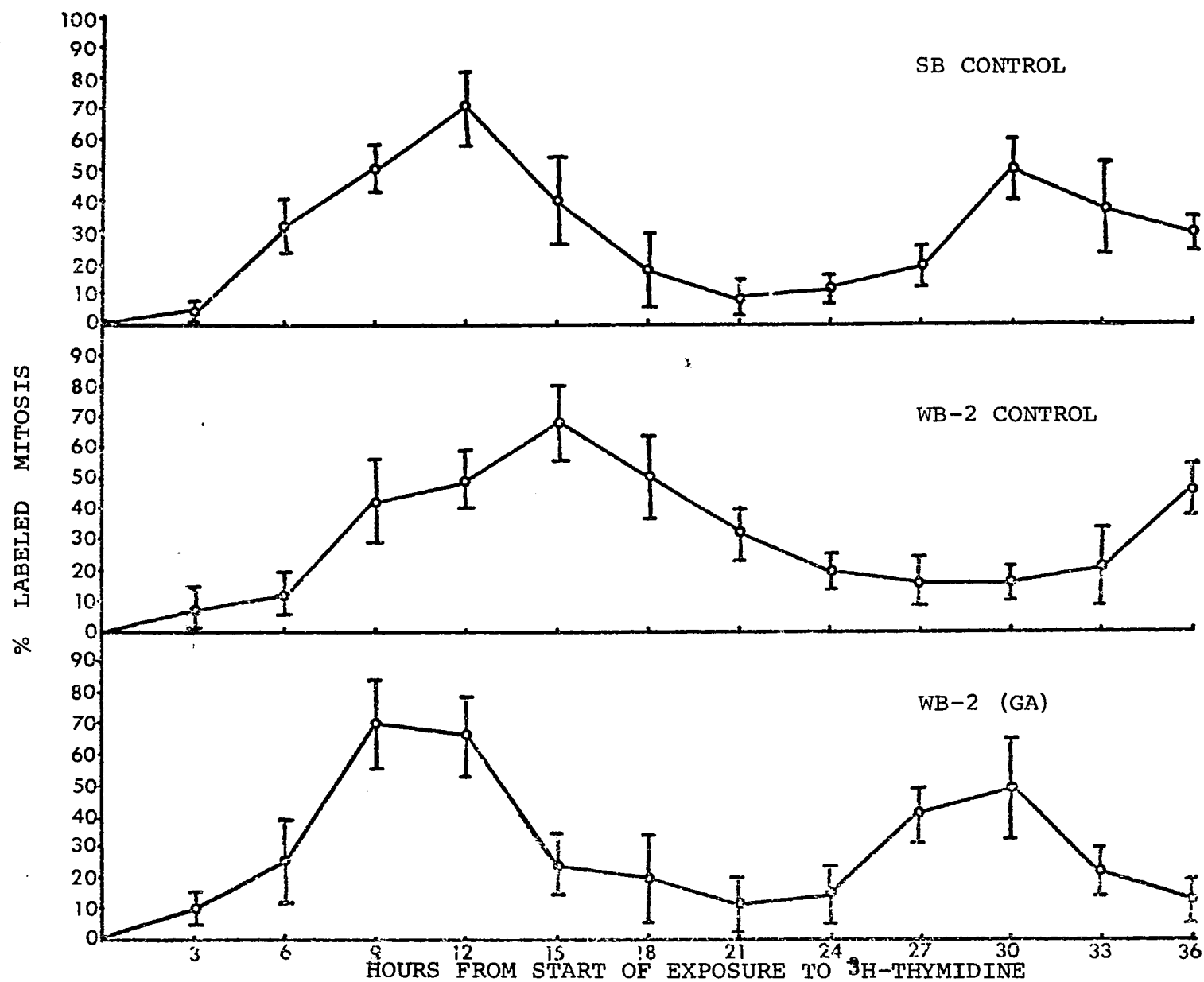


Figure 6. The percent of labeled mitosis following an hour exposure to ³H-thymidine in the shoot apical meristems of WB-2 SB and WB-2(GA) seedlings.

slightly decreased the duration of the G2 period in WB-2 dwarf to a stage which was comparable to that of SB control. However, the duration of S period in WB-2 dwarf was shortened by GA for 5.1 hours to a period which was 2.1 hours shorter than that of SB control. In this case, the shortening of the duration of cell cycle in WB-2 by treatment with GA was mainly due to a decrease in the duration of S period.

The cells with substantial nuclei in the meristematic region can be differentiated from elongating cells without distinguishable nuclei by the Feulgen stain. The comparisons of the meristematic zone in the shoot apex of SB control, WB-2 control, and WB-2 treated with GA are given in Table 2. The number of meristematic cells in WB-2 was less than that of SB and WB-2 treated with GA. However, the differences were not statistically significant by least significant difference at the 1% level. The number of cell files in the meristems of WB-2 control and WB-2 treated with GA were about equal, and were slightly lower than that of SB control. Again, they were not significantly different. The number of meristematic cells per file was similar in SB and WB-2 seedlings. GA slightly increased the number of meristematic cells per file in WB-2. On the other hand, the numbers of mitotic cells in the meristematic zone of SB and WB-2 treated with GA were significantly higher than that of WB-2 control. The mitotic index was estimated from the frequency of mitotic cells in the meristematic populations. The mitotic index in WB-2 was significantly lower than that of SB and WB-2 treated with GA.

Table 2. Number of meristematic and mitotic cells in a layer of middle section of shoot apical meristem in SB, WB-2 and WB-2(GA) seedlings

Treatment	No. of cells in apical meristem	No. of files in meristem	No. of cells per file in apical meristem	No. of mitotic cells (M)	Mitotic Index (MI) (MI=M/NCTx100)
SB control	*639.7 ^a	12.88 ^a	49.43 ^a	20.22 ^a	3.161 ^a
WB-2 control	584.3 ^a	11.87 ^a	49.22 ^a	10.54 ^b	1.804 ^b
WB-2(GA)	691.2 ^a	11.80 ^a	58.58 ^a	24.84 ^a	3.594 ^a
LSD 0.01	113.06	2.38	14.89	6.79	1.012

* each number represents the mean of data over the duration of cell cycle experiment.

** Means within a column followed by the same letter are not significantly different at P=0.01 according to LSD test.

Table 3. The estimation of cell production during a 90 hour period according to the meristematic activity.

Treatment	No. of cells in apical meristem	Duration of cell cycle	Cell proliferation rate per hour per file	Expected No. of cells produced per file during 90 hours	Actual No. of cells produced per file during 90 hours
SB control	49.43	21.3	2.32	208.9	188
WB-2 control	49.22	27.5	1.79	161.1	108
WB-2(GA)	58.58	18.5	3.17	285.0	283

Cell production during a given period can be estimated from two parameters: (1) the number of cells which are able to divide, and (2) the average duration of the cell cycle. Using the above information, an estimation of the number of cells produced during a 90-hour period was compared with the actual number of cells produced, as obtained from the previous cell number study (Table 3). The cell proliferation rate per hour in a file was obtained by dividing the number of meristematic cells per file by the duration of the cell cycle. The estimated number of cells produced during a 90-hour period was based on the average cell proliferation rate per hour x 90. The estimated numbers of cells produced in SB and WB-2 treated with GA were very similar to their respective actual numbers of cells produced during the 90-hour period examined. However, in the WB-2 control, estimated cell production was about 53 cells higher than the actual cell production during the 90-hour period.

DISCUSSION

DNA Synthesis and Cell Elongation

FUDR is a specific inhibitor of thymidylate synthetase (32), and when given at certain doses it can specifically block DNA synthesis in cells by arresting the formation of thymidine monophosphate from deoxyuridine monophosphate. Its specific effect on DNA synthesis may be verified by its reversibility with thymidine. In addition, the possible inhibition of RNA synthesis by FUDR through the conversion of FUDR to FU (5-fluorouracil) in vivo has also been reported (41). In this case, it is also necessary to apply uridine to the system in order to test whether the growth is blocked by the inhibition of RNA synthesis instead of DNA synthesis. The results with FUDR show that the growth of WB-2 seedlings was completely prevented by a 10^{-4} M concentration of FUDR, whereas 90% of growth in SB was inhibited by the same concentration of FUDR. The inhibitory effects in both lines could be reversed by thymidine but not by uridine. Thus, DNA synthesis is correlated with the seedling growth of both SB and WB-2. The positive correlation between inhibition of DNA synthesis and the inhibition of both cell elongation and cell division by FUDR in SB and WB-2 seedlings agrees with previous reports that inhibitors of DNA synthesis often block the elongation of non-dividing cells as well as cell division in the meristematic region of the shoot apex (9,63). In addition, the GA-stimulated growth of hypocotyls in WB-2, which involves an increase of both cell division

and cell elongation, is completely inhibited by FUDR, and the inhibition is reversed by thymidine. Similar results, in which the inhibition of DNA synthesis prevents the GA induction of cell elongation, have been observed in lentil epicotyl (63) and cucumber hypocotyl (21). Thus, the present results suggest that DNA is being synthesized during the elongation of the cells in the non-dividing tissue of both SB and WB-2 hypocotyls, and the prevention of DNA synthesis blocks cell elongation.

Nitsan and Lang (64) noted that at least part of newly synthesized DNA induced by GA in the elongating cells of lentil hypocotyl occurred in the nucleus. The newly synthesized nuclear DNA during cell elongation in non-dividing tissues could be due to: (1) endopolyploidy or (2) certain genes or blocks of genes could be duplicated for promotion of cell elongation. However, in their study, Nitsan and Lang found no evidence of endopolyploidy. In addition, increases in DNA might occur outside of the nucleus. Degani and Atsmon (20) found that the enhancement of DNA synthesis by GA in cucumber hypocotyl occurred mainly outside of the cell nucleus, probably in chloroplasts and mitochondria.

DNA Synthesis and Cell Cycle in Apical Meristem

The estimates of the length of the cell cycle and its component stages in the shoot apical meristem of watermelon seedlings reported here are similar to those obtained by Jacquard (36) in the pith rib meristem of Rudbeckia bicolor. However, they are generally longer than the values previously obtained from root meristems of several species at a temperature

of 30°C (62,85).

The G1 and G2 periods are longer in WB-2 than in SB, and the durations of these stages in WB-2 are restored to a length comparable to SB by 3.2×10^{-4} M GA. However, the duration of S period in WB-2 is reduced by the same concentration of GA to a stage even shorter than SB control. Thus, the main effect of GA on the cell cycle is to shorten the duration of the DNA synthesis or S period. It is not surprising that GA affects the durations of G1 and G2 as well as the S period, since GA could be activating or inducing several general metabolic enzymes functioning in each of these periods. The induction of several hydrolytic enzymes by GA in the dwarf pea internode (15), lentil epicotyl (74), avena internode (42), and in barley endosperm (37,56) have been reported. In view of the dramatic effect of GA on the S period, specific enzymes activities related to DNA synthesis, such as DNA polymerases, nucleoside kinases, nucleotidases, etc., may also be enhanced by GA.

Factors affecting the length of the S period as discussed in the literature review can be summarized into four categories: (1) amount of nuclear DNA, (2) structure of chromosomes, (3) number of replication units, and (4) whether DNA synthesis is continuous or discontinuous. Since the genome of WB-2 differs from SB mainly by a single mutant gene, the amount of nuclear DNA and the structure of chromosomes in WB-2 should be similar to normal strains. The differences in the duration of S period between SB and WB-2 could possibly be due to the number of replication units, or

whether DNA synthesis is continuous or discontinuous or both.

In addition, there is evidence that metabolic processes other than those involved in DNA synthesis, such as histone synthesis (62) and RNA synthesis (53), may also control the duration of S period and the rate of DNA synthesis. Thus, the effects on the duration of the S period by GA or the dwarf gene could be due to several factors but they are not necessarily mutually exclusive.

Cell Proliferation in the Apical Meristem

The actual numbers of cells produced per file during a 90 hour period in SB and WB-2 treated with GA were close to the numbers estimated from their respective cell cycles and numbers of cells per file (Table 3). However, the actual cell production in WB-2 control was much lower than the expected number. These results indicate that the number of proliferating cells in the apical meristems of SB and WB-2 treated with GA are nearly equal to the respective numbers of cells in the apical meristems, but that the proliferating cells of WB-2 are lower than the number of cells in the apical meristem. Clowes (18) reported that a fraction of the cells in the root meristem of corn were not dividing or were dividing but with a very long duration of the cell cycle. He called the specific region of non-dividing cells the quiescent center. A quiescent center or similar group of non-dividing cells might also exist in the apical meristem of WB-2, but not in the apical meristems of SB and WB-2 treated with GA since most or all of the cells

in those apical meristems are proliferating. Thus, it appears that GA promotes cell proliferation not only by decreasing the duration of the cell cycle, but also by inducing non-dividing cells in the apical meristem to divide.

The effects of kinetin and auxin on the durations of cell cycle have been reported in the root meristems of Pisum (83) and Vicia (53,54). However, the effects of auxin and kinetin at the concentrations employed were inhibitory, and as such, the results would not appear to relate to the normal endogenous function of these hormones. There apparently have been no previous studies on the effect of GA on the cell cycle in either roots or shoots.

The lower mitotic index in WB-2 control as compared to SB and WB-2 treated with GA (Table 2) appears due to two factors:

- (1) Since the durations of mitoses among the three treatments were about the same, but the durations of interphase in SB and WB-2 treated with GA were shorter than WB-2 control, more cells would be expected to be seen in the stage of mitosis in the meristems of SB and GA treated WB-2 than in the WB-2 control.
- (2) Since not all the cells in the WB-2 control are proliferating, this undoubtedly decreases the mitotic index.

SUMMARY

- (1) The dw-2 dwarf gene exerted its effect on hypocotyl length through a major reduction in cell number and a minor reduction in cell length. These dwarf hypocotyls were essentially restored to the normal phenotype with exogenous applications of GA₃.
- (2) A correlation was obtained between the inhibition of DNA synthesis by FUDR and the inhibition of cell elongation and cell division in SB and WB-2 seedlings. The inhibition of DNA synthesis by FUDR prevented GA-stimulation of both cell elongation and cell division in the WB-2 seedlings.
- (3) The cell cycle in the shoot meristem of SB seedlings was shorter than that of WB-2, mainly because of a reduction in the duration of the S period. GA treatment shortened the cell cycle in the WB-2 dwarf by a major reduction in the length of the S period and lesser effects on the G1 and G2 periods.
- (4) The sizes of meristematic regions among seedling of SB, WB-2 and WB-2 treated with GA were similar. However, a large fraction of non-dividing cells were found in the WB-2 meristem, but not in SB or GA-treated dwarf. Thus, GA promoted cell proliferation in the shoot meristem of WB-2 seedlings not only by decreasing the duration of the cell cycle, but also by inducing non-dividing cells to divide.

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